

AD-A130 520

PATHOGENESIS OF DENGUE VACCINE VIRUSES IN MOSQUITOES

1/1

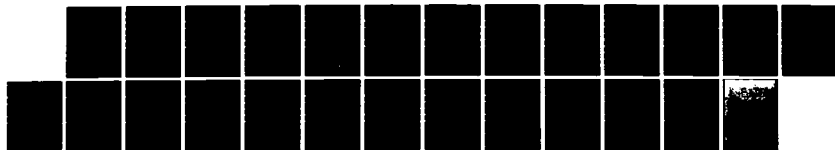
(U) YALE UNIV NEW HAVEN CONN SCHOOL OF MEDICINE

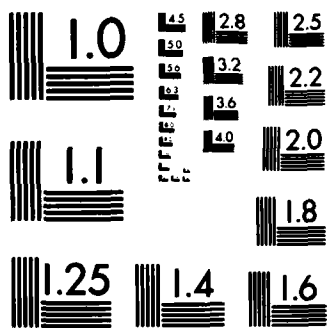
B J BEATY ET AL. 01 JAN 81 DAND17-79-C-9094

UNCLASSIFIED

F/G 6/5

NL





MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

AD A130520

12

UNCLASSIFIED

AD _____

PATHOGENESIS OF DENGUE VACCINE VIRUSES IN MOSQUITOES

Second Annual Report

Barry J. Beaty, Ph.D.
Thomas H.G. Aitken, Ph.D.

January 1, 1981

DTIC
ELECTE
JUL 20 1983
S D

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract DAMD17-79-C-9094

Yale University School of Medicine
New Haven, Connecticut 06510

Approved for public release; distribution unlimited.

The findings in this report are not to be construed
as an official Department of the Army position unless so
designated by other authorized documents.

DTIC FILE COPY

83 07 19 069

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-4130520	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) PATHOGENESIS OF DENGUE VACCINE VIRUSES IN MOSQUITOES		5. TYPE OF REPORT & PERIOD COVERED Annual progress report 1/1/80 - 1/1/81
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Barry J Beaty, Ph.D. Thomas H. G. Aitken, Ph.D.		8. CONTRACT OR GRANT NUMBER(s) DAMD17-79-C-9094
9. PERFORMING ORGANIZATION NAME AND ADDRESS Yale Arbovirus Research Unit, Yale University School of Medicine, 60 College Street New Haven, Connecticut 06510		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3M161102BS10.AA.063.
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE January 1, 1981
		13. NUMBER OF PAGES 24
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Dengue-2, S-1 vaccine, PR-159 parent, Comparative pathogenesis in mosquitoes, Reversion frequencies, immunofluorescence.		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) An artificial bloodmeal feeding technique was developed which resulted in infection rates of up to 100% in mosquitoes ingesting low passage or attenuated dengue-2 viruses.		

The technique was used in studies to compare the relative abilities of the PR-159 parent and S-1 vaccine dengue-2 viruses to infect and to be transmitted by Aedes albopictus and Aedes aegypti mosquitoes. The S-1 vaccine candidate virus was markedly less efficient than the PR-159 parent in infection of midgut cells, dissemination from midgut cells and infection of secondary target organs, and oral transmission by the vectors. In addition the vaccine strain replicated more slowly in the vectors than the parent strain.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



Accession For	
NTIS GRA&I	<input checked="checked" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By _____	
Distribution/ _____	
Availability Codes	
Dist	Avail and/or Special
A	

Summary

A technique was developed which resulted in consistent and efficient oral infection of mosquitoes with non-adapted dengue-2 viruses.

Studies were then conducted to compare the efficiency of oral infection, mode of development, and transmission potential of the dengue-2 S-1 candidate vaccine virus and the parent PR-159 virus in Aedes aegypti and Aedes albopictus mosquitoes. Both virus strains were capable of oral infection of the vector species. However, only 20% (18/90) of Ae. albopictus mosquitoes engorging the S-1 vaccine became infected while 57% (43/76) of those engorging the PR-159 strain became infected. This differential occurred even though the S-1 vaccine bloodmeal (6.3 log₁₀TCID₅₀/ml) titered almost 2 logs higher than the PR-159 bloodmeal (4.5 log₁₀TCID₅₀/ml). The same phenomenon was observed with Ae. aegypti; 40% (19/48) became infected with the S-1 vaccine, while 63% (15/24) became infected with a lower titered PR-159 blood meal preparation.

The S-1 vaccine developed more slowly in vectors than the parent PR-159 strain. Dengue-2 virus antigen was detected by immunofluorescence after 11-14 days extrinsic incubation in 35% (14/40) and 3% (1/40) of mosquitoes engorging the parent and vaccine strains respectively. After 15-22 days extrinsic incubation antigen was detected in 81% (29/36) of the mosquitoes that engorged the parent strain and only in 34% (17/50) of those infected with the vaccine strain. Again, the titer of the S-1 vaccine preparation was substantially higher than the parent PR-159 virus.

Both viruses were orally transmitted by infected Ae. albopictus. Seventy-nine percent (15/19) of those mosquitoes infected with the PR-159 strain transmitted, while 56% (9/16) transmitted the S-1 strain.

In order to determine reversion potential, the S-1 vaccine was sequentially passed in Ae. aegypti, Ae. albopictus, and Toxorhynchites amboinensis mosquitoes. The vaccine was also passed alternately in mosquitoes and mammalian cell cultures. These materials are currently being analyzed at WRAIR for possible S-1 vaccine reversion to virulence.

Table of Contents

	<u>Page</u>
DD Form 1473.....	2
Foreword.....	4
Summary.....	5
Table of Contents.....	6
Body of Report.....	7
I. Statement of the problem.....	7
II. Background.....	7
III. Approach.....	8
IV. Materials and Methods.....	8
A. Viruses.....	8
B. Mosquitoes.....	9
C. Conjugate.....	9
D. Virus assay.....	9
E. Oral infection of Mosquitoes.....	9
F. In vitro assay for oral transmission of dengue viruses...	10
V. Results.....	10
A. Replication of viruses after intrathoracic inoculation.....	10
B. Replication of viruses after oral infection.....	10
C. Oral transmission studies.....	11
D. Pathogenesis studies.....	11
E. Vaccine reversion studies.....	12
F. Development of techniques to efficiently orally infect vector mosquitoes.....	12
VI. Discussion.....	13
VII. Conclusions.....	14
Tables.....	16-21
References.....	22
Distribution List.....	24

I. Statement of the problem

The purpose of this research project is to determine if dengue parental and candidate vaccine viruses differ in their respective abilities to infect, to replicate in, and to be transmitted by Ae. aegypti and Ae. albopictus mosquitoes. Attenuated candidate vaccines and parental strains of dengue-1, dengue-2, and dengue-4 viruses will be compared in their vector-virus interactions.

The second, and related, objective of this research project is to determine if attenuated vaccine strains revert to virulence after mosquito passage. Should a live dengue vaccine be capable of infecting and subsequently be transmitted by mosquitoes to a new vertebrate and should the vaccine revert to virulence as a consequence of mosquito passage, then a natural infection cycle could be initiated.

The rationale for this project is that the temperature sensitive (ts) vaccine strains of the dengue viruses which are attenuated for man will also be modified in one or more parameters of vector-virus interactions. The hypotheses are 1) the vaccine strains will be less capable than parental strains of vector infection, 2) vaccine strains will differ from parent strains in their mode of development, 3) the vaccine strains will be less efficiently transmitted than parent strains, and 4) that the small plaque ts mutant virus populations will remain stable upon passage in vector mosquitoes.

II. Background

Dengue is of great tactical significance to the military because large numbers of troops can become incapacitated in a short period of time. Attenuated dengue vaccines have been developed at WRAIR.

The dengue-2 S-1 vaccine and PR-159 parent strains are the subject of this project report. The S-1 vaccine was derived from the serum of patient PR-159 of Puerto Rico (Eckels et al., 1976). The virus was passaged 6 times in Lederle certified African green monkey kidney cells. Passage 6 is designated the parent strain and S-1 represents the progeny of a small plaque derived from the parent strain. The S-1 clone is ts, titers 340 times higher in LLC-MK₂ cells than in mice. does not produce viremia in rhesus monkeys, produces barely detectable viremia in chimps and in man. Only 1 of 150 Ae. aegypti mosquitoes fed on volunteers became infected, but it did not transmit the virus after 21 days extrinsic incubation.

Ideally a vaccine should not produce viremia, but if it does, it is reasonable to expect that the vaccine strain will infect mosquitoes poorly and will be inefficiently transmitted. This was demonstrated with the 17D yellow fever vaccine (Roubaud et al., 1937; Whitman, 1939), French neurotropic yellow fever vaccine (Davis et al., 1932; Roubaud and Stefanopoulo, 1933; Peltier et al., 1939), mouse-adapted dengue type 1 (Sabin, 1948), and African green monkey kidney-adapted dengue type 2 (Price, 1973). Sabin (1948) showed that attenuated dengue, passed through mosquitoes, did not revert to pathogenicity for man.

Thus, even if the vaccine did develop sufficient viremia to infect vectors, there would be little likelihood that the virus would be transmitted and that it would revert to virulence.

III. Approach

The working hypothesis was made that the ts candidate vaccine viruses and the parental wild-type viruses would behave differently in vector mosquitoes. To test this hypothesis the efficiency of oral infection of each parental and vaccine candidate strain was to be determined in dose response studies. Sequential 10-fold dilutions of the virus preparations were to be used to infect groups of a minimum of 10 sibling mosquitoes per dilution. Such studies would also provide information about the optimal infective dose for the transmission and pathogenesis studies; doses much greater than the threshold could obscure differences in infectivity between the vaccine and parental viruses.

Mosquitoes for studies to determine infection rates, extrinsic incubation periods, and rates of oral transmission were to be infected via engorgement on known titer blood-virus mixtures. In the event of low infection rates, several animal models were to be investigated.

Vector-virus interactions were to be further investigated using immunofluorescent techniques to localize antigen in situ in organ dissections and cryostat sections of infected mosquitoes. The sites of restriction of replication (if restriction exists) of the vaccine strains would be defined by the comparative IF studies of antigen development in organs of mosquitoes.

The combination of transmission and comparative pathogenesis studies and the determination of dose-response curves should be adequate to reveal differences in vector-virus interactions between parental and vaccine viruses. In order to implement the proposed research it was necessary to develop a technique which would permit efficient oral infection of mosquitoes by parent and vaccine viruses.

Vector competence studies and especially dose-response studies are greatly facilitated by the use of artificial bloodmeals. Unlike a viremic host, a known titer of virus can be presented to the mosquitoes and sequential dilutions of virus can be prepared for dose-response studies. Unfortunately, it is necessary to prepare blood virus mixtures with extremely high titers in order to obtain the same mosquito infection rate as would be obtained if the mosquitoes fed on a host with a much lower titered viremia. This is indicative that an unnatural infection route is being utilized by the virus which may not be pertinent to field circumstances. Studies were conducted with other types of blood meal preparations and viremic hosts to find a more satisfactory infection mechanism. The isolation of dengue and yellow fever virus from leukocytes (Wheelock and Edelman, 1969; Halstead et al., 1977; Marchette and Halstead, 1978) suggested that one or more of the white cells may function to promote mosquito midgut infection.

IV. Methods and Materials

A. Viruses:

Stock viruses for both the parental and S-1 vaccine strains of dengue-2 virus were prepared in LLC-MK₂ cells. The original infected human serum (PR-159) was the source of the parental virus. The experimental vaccine virus (Lot #4, Jan. 1976, WRAIR) was the seed for the vaccine stocks. To prepare the tissue culture stock pools, monolayers of LLC-MK₂ cells were inoculated with the respective seed

virus. On day 6 post inoculation, fluids were harvested, centrifuged, and the supernatant was aliquoted and frozen.

B. Mosquitoes:

Two colonized strains of Ae. aegypti and 2 colonized strains of Ae. albopictus were used in these studies.

Ae. aegypti - Santo Domingo
New Orleans

Ae. albopictus - Jakarta
Oahu

The mosquitoes were maintained at 27°C, 65-75% RH in screened $\frac{1}{2}$ pt ice cream cartons and provided with 10% sucrose.

C. Conjugate:

The anti-dengue-2 conjugate was prepared by hyperimmunization of mice (Brandt et al., 1967). Immunoglobulins were precipitated from the ascitic fluids with $(\text{NH}_4)_2\text{SO}_4$ and conjugated with fluorescein isothiocyanate (Spendlove, 1966; Hebert et al., 1972). Conjugated antibodies were purified by Sephadex G-50 column chromatography. The conjugate titered 1:32 and was used at 1:16.

D. Virus Assay:

Titration - Serial 10-fold dilutions of infectious bloodmeals were inoculated into 8 well Lab-Tek slides seeded with BHK-21 cells. Four days post-inoculation the slides were examined for viral antigen by IF.

Antigen detection - IF was used to localize viral antigen in situ in organ dissections and cryostat sections of mosquitoes (Beaty and Thompson, 1976, 1978) and in head and abdominal squash preparations (Kuberski and Rosen, 1977).

Oral transmission - Transmission experiments were carried out by the technique of Beaty and Aitken (1979) in which mosquito saliva was collected in capillary tubes containing a measured amount of diluent. The saliva was then assayed in either Ae. triseriatus males or in Ae. aegypti by intrathoracic inoculation.

E. Oral Infection of Mosquitoes

Considerable effort was devoted to development of an effective technique for oral infection of mosquitoes with low passage or vaccine strains of dengue (see page 11). After much experimentation, the following protocol was developed.

Parental and vaccine viruses were each inoculated into flasks of LLC-MK₂ or Ae. albopictus C6/36 cells. Seven to 10 days post-inoculation cells were detached from the flasks with rubber policemen, and the cell fluid suspensions were centrifuged at 500xg for 10 minutes. The cell pellet was resuspended in 1 ml of the remaining fluid, and combined with 1 ml of washed human red blood cells

and	-9-	0.5	ml
-----	-----	-----	----

of 10% sucrose in heat-inactivated calf serum. Drops of this artificial bloodmeal were placed on the screening of cages holding mosquitoes. Engorged mosquitoes were removed and maintained. All mosquitoes were held at 27°C and 65-75% RH for 14-24 days.

F. In vitro Assay for oral transmission of dengue viruses:

This laborious technique was necessitated by the lack of a small laboratory animal model susceptible to the low passage PR-159 virus and the attenuated S-1 virus.

After 14-24 days extrinsic incubation, mosquitoes were starved overnight prior to the transmission attempt. Capillaries were charged with 0.0075 ml of a 10% FCS-3% sucrose - 10 mM ATP solution. Mosquitoes were cold anesthetized, wings and the anterior 4 legs were removed, and the proboscis was inserted into the capillary. Mosquitoes were allowed to engorge for 1 hour before they were removed, and heads and abdomens were severed and squashed on slides. The smears were stained with the anti-dengue conjugate and examined for the presence of viral antigen using a Leitz-Wetzlar microscope with an HBO Osram 200W mercury vapor bulb and a KP 490-K510 filter system. After engorgement the contents of the meal were promptly inoculated intrathoracically into 10 recipient mosquitoes. After 10-14 days incubation, recipient mosquitoes were processed by the head squash IF procedure (Kuberski and Rosen, 1977).

V. Results

A. Replication of viruses after intrathoracic inoculation:

In order to determine the relative ability of the PR-159 and S-1 strains to replicate in the Ae. aegypti and Ae. albopictus, mosquitoes were intrathoracically inoculated with sequential dilutions of the respective stock viruses. At 7 and 14 days incubation, mosquitoes were assayed for the presence of viral antigen by IF. The series of dilutions did not reach an endpoint (Table 1), but apparent differences in efficiency of replication were demonstrated. For the Ae. aegypti infected with the PR-159 strain, viral antigen was detected in 70% (32/46) of the mosquitoes examined after 7 days extrinsic incubation. However, only 30% (14/46) of those infected with the S-1 strain contained detectable antigen. At 14 days extrinsic incubation, virtually all of the mosquitoes examined contained detectable viral antigen. Thus, the S-1 strain seemed to replicate more slowly in the vector than the PR-159 strain. Studies of the growth curves of the respective viruses are currently being conducted to determine if the differences in viral antigen accumulation correlate with differences in quantities of infectious virus.

B. Replication of viruses after oral infection:

Studies were conducted to determine the efficiency of each virus to orally infect Ae. aegypti and Ae. albopictus. Both virus strains were capable of oral infection of the two vector species. Infection rates for Ae. aegypti were 63% (15/24) and 40% (19/48) for the PR-159 and S-1 strains respectively (Table 2). Infection rates for Ae. albopictus were 38% (15/39) and 24% (10/42) for the parent and vaccine strains respectively. In each mosquito strain (Table 2), the S-1 vaccine seemed less efficient in establishment of oral infection of mosquitoes than the parent PR-159 dengue virus.

To investigate further this phenomenon, Ae. albopictus mosquitoes (Oahu) were permitted to engorge a bloodmeal containing either the PR-159 or the S-1 virus strain. Mosquitoes from each group were processed approximately daily for 3 weeks (Table 3). Heads and abdomens were severed and squashed on slides. The resultant smears were examined by IF for the presence of viral antigen. This protocol permitted a comparison of the PR-159 and S-1 strain in their relative abilities to: 1) infect Ae. albopictus, 2) replicate to detectable levels, and 3) escape from the midgut into the hemocoel and establish a disseminated infection. If antigen was detected only in the abdominal squash, it was presumed to be restricted to midgut tissues. If antigen was detected in the headsquash, the midgut infection had occurred and virus had subsequently disseminated from the midgut.

By 14 days extrinsic incubation, 35% (14/40) of the mosquitoes engorging the PR-159 virus contained detectable antigen in the midgut. In contrast, only 3% (1 of 40) of those ingesting the S-1 strain contained detectable antigen. After 15-22 days incubation, 81% (29/36) of the mosquitoes contained detectable PR-159 antigen, but only 34% (17/50) contained detectable quantities of the S-1 strain. Importantly, these differences in infectivity occurred even though the infectious titer of the PR-159 parent strain was approximately two logs less than that of the S-1 vaccine virus (Table 3).

The PR-159 strain was also more efficient in establishing disseminated viral infections. At 15-22 days extrinsic incubation, 69% (20/29) and 35% (6/17) respectively of the PR-159 and S-1 infected Ae. albopictus contained detectable viral antigen in the headsquash (Table 3). Likewise, the PR-159 strain seemed to replicate more efficiently than the S-1 strain. Viral antigen became detectable earlier in both the head and abdominal squashes of those mosquitoes engorging the PR-159 virus.

These data correlate well with the results obtained in the intrathoracic infection studies. While both the PR-159 and S-1 vaccine viruses are capable of infecting Ae. albopictus and establishing disseminated infections, the vaccine is notably less efficient. In addition the S-1 strain seems to replicate in mosquito tissues at a much slower rate than the parent strain.

C. Oral transmission studies:

Since both viruses were capable of establishing disseminated infections in Ae. albopictus, studies were conducted to determine the transmission potential of the two virus strains. Ae. albopictus mosquitoes were orally infected with either the PR-159 or the S-1 vaccine strain as described previously. After 21-24 days extrinsic incubation, mosquitoes were assayed for transmission ability using the in vitro technique (see Methods). The numbers of mosquitoes that became infected and transmitted are shown in Table 4. The parent strain was again markedly more efficient in infection of Ae. albopictus. The titers of the two virus preparations were equal (Table 4). This resulted in infection of 100% (20/20) of the mosquitoes engorging the PR-159 strain, but only 55% (16/29) of those engorging the S-1 strain. The parent strain also seemed to be more efficiently transmitted; 75% (15/20) transmitted the parent compared to 56% (9/16) that transmitted the vaccine strain.

D. Pathogenesis studies:

Preliminary studies were conducted to determine the mode of development of the viruses in Ae. albopictus. IF technique was used to detect parent and vaccine viral antigen in situ in organ systems and tissues of infected mosquitoes. In initial studies only headsquash positive mosquitoes (either parent or vaccine) were dissected. In both groups, viral antigen was detected in midguts, salivary glands, and ventral nerve cords. Both viruses were widely distributed in tissues of orally infected Ae. albopictus. This methodology will now be applied in comparative pathogenesis studies of the two viruses and will hopefully permit determination of the anatomic basis for the observed differences of the S-1 vaccine and PR-159 parent dengue-2 viruses in mosquitoes.

E. Vaccine reversion studies:

A major goal of this research is to determine if the S-1 candidate vaccine virus will revert to virulence as a consequence of mosquito passage. To determine reversion potential, the S-1 vaccine virus was sequentially passed 4 times in Ae. aegypti, Ae. albopictus, and Toxorhynchites amboinensis mosquitoes by intrathoracic inoculation. The S-1 virus was also alternately passed 3 times in LLC-MK₂ cells and Ae. aegypti mosquitoes to more closely approximate the natural circumstances of arbovirus transmission. These materials have been triturated, coded, and forwarded to Dr. Kenneth Eckels at the WRAIR for examination for plaque size and temperature sensitivity.

F. Development of techniques to efficiently orally infect vector mosquitoes:

During the previous granting period, considerable time and effort was expended in developing an efficient technique to orally infect Ae. aegypti and Ae. albopictus mosquitoes. High titered virus stocks were prepared in mosquitoes, LLC-MK₂, J-111 and BHK-21 vertebrate cell lines and the C6/36 clone of Igarashi's Ae. albopictus cell line. Frozen virus stocks were thawed and mixed with 10% sucrose in either defibrinated blood or washed human red blood cells. Even with these high titered virus-blood preparations, only a small percentage of engorging mosquitoes became infected.

Brandt et al. (1980), Halstead and O'Rourke (1977) and others demonstrated that very dilute homologous or more concentrated heterologous antibody complexes with dengue virus in a non-neutralizing manner. This antibody apparently provides the virus with a molecular ride into circulating leukocytes via the Fc receptor. Although no one has demonstrated Fc receptors on mosquito mesenteron cells we postulated that, since virus in viremic human blood is highly infectious to mosquitoes the presence of non-neutralizing dengue antibody and leukocytes would enhance oral infection of mosquitoes (cell lines provided by Dr. C.J. Peters, USAMRIID, Dr. Walter Brandt, and Dr. K. Eckles of WRAIR). Bloodmeals were prepared with different concentrations of homologous and heterologous antibody and cells mixed with a thawed virus preparation grown in LLC-MK₂ cells. Again the results were not encouraging; few mosquitoes became infected.

We prepared a crude approximation of viremic human blood to use to infect mosquitoes. Whole blood was drawn from one of us (with yellow fever antibodies) and mixed with a minimal amount of heparin. This was added to a flask of C6/36 cells which had been previously (7 days) infected with PP-159 virus. A control flask contained infected C6/36 cells only. Four days later the cells (rbc's,

leukocytes and C6/36 cells) were mechanically removed and fed to mosquitoes. Washed rbc's were added to the control flask and fed to mosquitoes. After an extrinsic incubation period of 14 days, the mosquitoes were examined for the presence of viral antigen. Over 20% of the mosquitoes were infected, the highest rate so far obtained. Subsequently the technique was modified to that described in the Methods Section (page 4). Mosquito oral infection rates of up to 100% (Table 4) were achieved, depending upon the virus titer of the tissue culture preparation. Several hypothesis could account for the success of the unfrozen virus meal preparations: 1) freezing virus preparations might somehow alter glycoprotein conformation rendering the virus less capable of interacting with midgut cell receptors, 2) virus in cells might be protected from proteolytic enzymes or other toxic substances, 3) cell or membrane associated virus might better interact with midgut cells. A pilot study was conducted to test these hypothesis. Parent dengue 2 virus was used to infect C6/36 cells. After 7 days incubation, cells were scraped from the flask and separated by centrifugation (800xg, 30 minutes). The infected cells and supernatant were split into 2 lots, one was held at room temperature while the other was frozen and thawed 3 times. These 4 preparations were mixed with human red blood cells and 10% sucrose in serum and fed to Ae. albopictus mosquitoes. Infection rates (Table 5) ranged from 100% for mosquitoes ingesting unfrozen virus-infected cells to 72% for mosquitoes ingesting frozen supernatant virus. These initial results are difficult to interpret: 1) the centrifugation regimen was inadequate to remove membrane associated virus from the "cell-free" supernatant and 2) quick-freezing and thawing is probably not analagous to long term storage of virus stocks at -70°C. Nonetheless, in each comparison, the unfrozen virus preparation resulted in higher infection and subsequent dissemination rates than the frozen virus preparations. To determine the generality of this phenomenon, similar studies were conducted with La Crosse (LAC) virus, family Bunyaviridae, and its vector, Ae. triseriatus. Mosquitoes were fed meals of washed human rbc's, 10% sucrose in calf serum and 1) a frozen infected mouse brain suspension, 2) infected cell culture supernatant, and 3) infected BHK-21 cells. A fourth infectious source was viremic suckling mice. Virus titers ranged from 5.5 to 6.8 log₁₀TCID₅₀/ml (Table 6). Engorged mosquitoes were examined by IF for viral antigen 14 days post-feeding.

Fifty percent of the mosquitoes that fed on a viremic mouse circulating 5.5 log₁₀TCID₅₀/ml of LAC virus became infected (Table 6). In contrast, of those mosquitoes ingesting a meal containing 6.0 to 6.5 log₁₀ TCID₅₀/ml of a frozen mouse brain virus preparation, only 3% (1/38) became infected. Those mosquitoes engorging an artificial meal containing either cell culture supernatant virus or infected cells had 30% (12/40) and 49% (17/35) infection rates respectively. Interestingly those mosquitoes ingesting the unfrozen virus preparations had higher disseminated infection rates than those mosquitoes feeding on the viremic mouse.

VI. Discussion

The use of unfrozen cell-associated dengue virus bloodmeal preparations would seem to be a satisfactory substitute for a viremic host for mosquito infection studies. Depending on the virus titer in the cell cultures, oral infection rates of up to 100% (Table 4) were obtained. In previous dengue-2 studies using frozen stock virus preparations, we were only able to infect a very small percentage of

the engorging mosquitoes. The basis for the enhanced infectivity of the unfrozen virus strains is as yet undetermined, but since the phenomenon was also observed with a Bunyavirus, it may be applicable to other enveloped viruses.

The S-1 vaccine seemed to be markedly less efficient than the parent PR-159 strain in interactions with potential vector species. The S-1 vaccine replicated more slowly than the PR-159 strain after intrathoracic inoculation (Table 1); it was considerably less efficient in oral infection of vectors (Table 2 and 3); it was considerably less efficient in developing a disseminated infection (Table 3); and when disseminated infection did occur, it was later than that for the PR-159 strain. The S-1 vaccine strain was also less efficiently transmitted than the parent strain (Table 4). Thus, the S-1 vaccine strain attenuated for humans would also seem to be modified in its ability to interact with the colonized vector mosquitoes used in these studies. In fact, the experimental design of this research undoubtedly lessened real differences between the S-1 vaccine and the PR-159 parent strains. The blood meal preparations titered approximately 5.5 to 7.5 \log_{10} TCID₅₀/ml for both virus strains. While such viremia titers would be expected for wild type dengue-2 virus, the S-1 vaccine virus produces a barely detectable viremia. Thus, it would be unlikely that 1) if a mosquito engorged it would become infected, 2) if a mosquito became infected it would develop a disseminated infection, and 3) if a mosquito developed a disseminated infection it would be capable of orally transmitting the virus.

However, it must be emphasized that the majority of these observations were made using highly laboratory adapted strains of mosquitoes. Since genetic variability in vector competence of Ae. aegypti and Ae. albopictus populations has been demonstrated (Gubler and Rosen, 1976, Gubler et. al., 1979), similar studies should be conducted with selected, epidemiologically significant geographic strains of these mosquitoes. In addition, oral transmission studies have not yet been done with Ae. aegypti mosquitoes. While it would seem likely that differences in transmission capability for the parent and vaccine strains would exist between Ae. aegypti and Ae. albopictus, such studies are, nonetheless, indicated.

On the basis of these preliminary studies, the S-1 vaccine strain would seem to be sufficiently modified (in its ability to infect and to be transmitted by vector mosquitoes) to preclude secondary infections as a result of mosquitoes becoming infected by feeding on recent vaccinees. Since dissemination of S-1 infection in Ae. albopictus was not detected until 19 days post engorgement, in the unlikely event that a mosquito became infected, it would probably die before it was capable of transmission. The question of vaccine reversion to virulence remains to be answered.

VII Conclusions

1. The unfrozen, cell-associated virus preparation is a satisfactory technique for oral infection of mosquitoes with low passage or attenuated vaccine strains of dengue-2 virus.

2. The S-1 candidate vaccine dengue-2 virus was less efficient than the PR-159 parent virus in the following vector-virus interactions:

- a) Replication in intrathoracically infected mosquitoes.

- b) Oral infection of vector mosquitoes.
- c) Dissemination of infection from vector midgut cells and infection of secondary target organs.
- d) Oral transmission by the vector.

Table 1. Infection rates of Aedes aegypti (SDO) mosquitoes inoculated with dengue 2 parent and vaccine viruses.

Virus dilution inoculated	Incubation period (days)	Dengue 2 virus	
		Parent (%) ^a	Vaccine (%) ^b
10 ⁰	7	11/12 ^c (92)	3/12 (25)
10 ⁻¹	7	7/10 (70)	4/12 (33)
10 ⁻²	7	6/12 (50)	6/10 (60)
10 ⁻³	7	8/12 (67)	1/17 (8)
Total		32/46 (70)	14/46 (30)
10 ⁰	14	14/15 (93)	15/15 (100)
10 ⁻¹	14	8/8 (100)	15/15 (100)
10 ⁻²	14	----	15/15 (100)
10 ⁻³	14	16/16 (100)	4/5 (80)
Total		38/39 (97)	49/50 (98)

^a 6.3 log₁₀ TCID₅₀/ml 10⁰ dilution.

^b 7.0 log₁₀ TCID₅₀/ml 10⁰ dilution.

^c No. positive over number tested by IF.

Table 2. Infection rates of mosquitoes fed on dengue 2 parent and vaccine viruses.^a

Mosquito species (<u>strain</u>)	Dengue 2 virus	
	Parent (%) ^b	Vaccine (%) ^c
<u>Aedes aegypti</u> (Santo Domingo)	7/15 (47) ^d	8/26 (31)
<u>Aedes aegypti</u> (New Orleans)	8/9 (89)	11/22 (50)
Total <u>Aedes aegypti</u>	15/24 (63)	19/48 (40)
<u>Aedes albopictus</u> (Jakarta)	10/34 (29)	1/26 (4)
<u>Aedes albopictus</u> (Oahu)	5/5 (100)	9/16 (56)
Total <u>Aedes albopictus</u>	15/39 (38)	10/42 (24)
Total <u>Ae. aegypti</u> incl. <u>Ae. albopictus</u>	30/63 (48)	29/90 (32)

^aMosquitoes were fed a suspension of infected C₆/36 cells, washed human red blood cells, calf serum and sucrose.

^b5.5-6.3 log₁₀ TCID₅₀/ml.

^c5.5-7.0 log₁₀ TCID₅₀/ml.

^dNumber positive/number tested by IF.

Table 3. Infection rates of Aedes albopictus (OAHU) mosquitoes fed on dengue 2 parent and vaccine viruses.^a

Extrinsic incubation (days)	Dengue 2 virus	
	Parent ^b	Vaccine ^c
11	4/10 (1/10) ^d	0/10 (0/10)
12	4/10 (0/10)	0/10 (0/10)
13	1/10 (0/10)	0/10 (0/10)
14	5/10 (0/10)	1/10 (0/10)
15	7/9 (3/9)	1/10 (0/10)
19	8/9 (6/9)	2/10 (1/10)
20	7/10 (6/10)	2/10 (1/10)
21	7/8 (5/8)	4/10 (1/10)
22	-- --	8/10 (3/10)
Total (%)	43/76 21/43 (57) (49)	18/90 6/18 (20) (33)

^a Mosquitoes were fed a suspension of infected Aedes albopictus cells (clone C6/36), washed human red blood cells, calf serum and sucrose.

^b 4.5 log₁₀ TCID₅₀/ml.

^c 6.3 log₁₀ TCID₅₀/ml.

^d No. positive by IF over no. tested. Ratio in parenthesis reflects virus antigen disseminated to head tissues.

Table 4. Infection and transmission rates for Aedes albopictus
(OAHU) orally infected with dengue 2
parent and vaccine viruses.^a

	Dengue 2 virus ^b	
	Parent	Vaccine
No. mosquitoes exposed	20	29
No. infected (%)	20 (100)	16 (55)
No. transmitting ^c (%)	15 (75)	9 (56)

^aExtrinsic incubation period was 21-24 days.

^bEach blood meal contained 7.3 log₁₀ TCID₅₀/ml post-feeding.

^cIn vitro transmission technique of Aitken, 1977.

Table 5. Infection rates for Aedes albopictus (OAHU) ingesting dengue 2 (PR-159) virus.^a

Unfrozen				Frozen ^b			
Cell associated Virus		Supernatant Virus		Cell associated Virus		Supernatant Virus	
Infected(%)	Disseminated ^d (%)	Infected(%)	Disseminated(%)	Infected(%)	Disseminated(%)	Infected(%)	Disseminated(%)
21/21 ^c (100)	16/21 (76)	29/30 (97)	25/30 (83)	26/31 (84)	19/31 (61)	21/29 (72)	11/29 (38)

^a Meals consisted of 1ml virus preparation grown in Aedes albopictus C6/36 cells, 1 ml washed human rbc and 0.5 ml 10% sucrose in calf serum.

^b Frozen and thawed 3 times prior to use.

^c No. positive over no. tested by IF.

^d Viral antigen detected in head tissues.

Table 6. Infection rates of Aedes triseriatus mosquitoes fed on various La Crosse virus preparations.

Meal ^b	Titer ^c	Infection rate (%) ^a	
		Gut	Disseminated ^e
Frozen virus ^d	6.0-6.5	1/38 (3)	1/38 (3)
cell culture supernatant	5.5-6.3	12/40 (30)	8/40 (20)
cells & supernatant	6.0-6.8	17/35 (49)	10/35 (29)
viremic suckling mice	5.5	8/16 (50)	1/16 (6)

- ^a No. positive over no. tested for viral antigen by FA after 14 days extrinsic incubation.
- ^b Meals consisted of virus preparations, washed human red blood cells, calf serum and sucrose.
- ^c Log₁₀ TCID₅₀/ml.
- ^d Suckling mouse brain preparation; also used to infect BHK-21 cells and inoculate suckling mice for viremic meal.
- ^e Virus antigen detected by FA in head tissues.

References:

- Beaty, B.J. and Aitken, T.H.G. 1979. In vitro transmission of yellow fever virus by geographic strains of Aedes aegypti. Mosquito News, 39: 232-238.
- Beaty, B.J. and Thompson, W.H., 1976. Delineation of La Crosse virus in developmental stages of transovarially infected Aedes triseriatus. Am J. Trop. Med. & Hyg. 25: 505-512.
- Beaty, B.J. and Thompson, W.H. 1978. Tropisms of La Crosse (California encephalitis) virus in bloodmeal-infected Aedes triseriatus (Diptera: Culicidae). J. Med. Entomol., 14: 499-503.
- Brandt, W.E., McCown, J.M., Top. F.H., Bancroft, W.H., and Russell, P.K. 1979. Effect of passage history of dengue-2 virus replication in subpopulations of human leukocytes. Inf. Immun. 26: 534-541.
- Brandt, W.E., Buescher, E.L., and Hetrick, F.M. 1967. Production and characterization of arbovirus antibody in mouse ascitic fluid. Am. J. Trop. Med. & Hyg. 16: 339-347.
- Davis, N.D., Lloyd, W., and Frobisher, M., Jr. 1932. Transmission of neurotropic yellow fever virus by Stegomyia mosquitoes. J. Exp Med. 56: 853-865.
- Eckels, K.H., Brandt, W.E., Harrison, V.R., McCown, J.M., and Russell, P.K. 1976. Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. Inf. Immun. 14: 1221-1227.
- Gubler, D.J. and Rosen, L. 1976. Variation among geographic strains of Aedes albopictus in susceptibility to infection with dengue viruses. Am. J. Trop. Med. & Hyg. 25: 318-325.
- Gubler, D.J., Nalim, S., Tan, R., Saipan, H., and Saroso, J. 1979. Variation in susceptibility to oral infection with dengue viruses among geographic strains of Aedes aegypti. Am. J. Trop. Med. & Hyg. 28: 1045-1052.
- Halstead, S.B., O'Rourke, E.J. 1977. Antibody enhanced dengue virus infection in primary leukocytes. Nature 265: 739-741.
- Halstead, S.B., O'Rourke, E.J., and Allison, A.C. 1977. Dengue virus and mononuclear phagocytes. II. Identity of blood and tissue leukocytes supporting in vitro infection. J. Exp. Med. 146: 218-229.
- Hebert, A., Pittman, B., McKinney, R.M., and Cherry, W.B. 1972. The Preparation and Physiochemical Characterization of Fluorescent Antibody Reagents, U.S.P.H.A. Bureau of Laboratories, Atlanta, Georgia.
- Kuberski, T.T. and Rosen, L. 1977. A simple technique for the detection of dengue antigen in mosquitoes by immunofluorescence. Am. J. Trop. Med. & Hyg. 26: 533-537.

Marchette, N.J., and Halstead, S.B. 1978. Phytohemagglutinin enhancement of dengue-2 virus replication in nonimmune rhesus monkey peripheral leukocytes. *Inf. & Imm.* 19: 40-45.

Peltier, M., Durieux, C., Jonchere, H., and Arquie, E. 1939. La transmission par pique de Stegomyia, du virus amaril neurotrope present dans le sang des personnes recemment vaccinees est-elle possible dans les regions ou ce moustique existe en abundance? *Rev. d'immunol.* 5: 172-195.

Price, W. 1973. Dengue-2 attenuated New Guinea C strain and attenuated JE (G9473). WRAIR Symposium on Determinants of Arbovirus Virulence, 13-14 December 1973.

Roubaud, E. and Stefanopoulo, G.J. 1933. Recherches sur la transmission par la voie stegomyienne du virus neurotrope murin de la fièvre jaune. *Bull. Soc. Path. exot.* 26: 305-309

Roubaud, E., Stefanopoulo, G.J., and Findlay, G.M. 1937. Essais de transmission par les stegomyies du virus amaril de cultures en tissu embryonnaire. *Bull. Soc. Path. Exot.* 30: 581-583.

Sabin, A.B. 1948. Dengue in "Viral and Rickettsial Infections of Man" Rivers T.M. ed. Lippincott, Philadelphia.

Spendlove, R.S. 1966. Optimal labeling of antibody with fluorescein isothiocyanate. *Proc. Soc. Exp. Biol. Med.*, 122: 580-583.

Vier, E. 1978. Comparative infectability of Aedes aegypti and Aedes albopictus with viruses from dengue fever and dengue hemorrhagic fever patients. MPH Essay, Yale University.

Wheelock, E.F., and Edelman, R. 1969. Specific role of each human leukocyte type in viral infections: II. 17-D yellow fever virus replication and interferon production in homogenous leukocyte cultures treated with phytohemagglutinin. *J. Immunol.* 103: 429-436.

Whitman, L. 1939. Failure of Aedes aegypti to transmit yellow fever cultured virus (17D). *Am. J. Trop Med.* 19: 19-26.

DISTRIBUTION LIST

12 copies

Director
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
ATTN: SGRD-UWZ-C
Washington, D.C. 20012

4 copies

Commander
U.S. Army Medical Research &
Development Command
ATTN: SGRD-RMA
Ft. Detrick, MD 21701

12 copies

Defense Technical Information
Center (DTIC)
ATTN: DTIC-DDA
Cameron Station
Alexandria, VA 22314

1 copy

Dean
School of Medicine
Uniformed Services University
of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20014

1 copy

Commandant
Academy of Health Sciences, US Army
ATTN: AHS-CDM
Fort Sam Houston, TX 78234

END

FILMED

8-83

DTIC